The conserved zinc finger protein VAB-23 is an essential regulator of epidermal morphogenesis in Caenorhabditis elegans

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The conserved zinc finger protein VAB-23 is an essential regulator of epidermal morphogenesis in *Caenorhabditis elegans*

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ABSTRACT

Caenorhabditis elegans is an excellent model to observe cell movements and shape changes during the morphogenesis of the egg-shaped embryo into an elongated tube-like larva. Although much is known about the structural determinants involved in epidermal morphogenesis, relatively little is known about the transcriptional and post-transcriptional regulatory networks involved.

Here, we describe the identification and functional characterization of the novel nuclear protein VAB-23, which belongs to a conserved protein family found in all metazoans. *C. elegans* VAB-23 is essential for ventral closure and elongation of the embryo. Time-lapse analysis indicates that VAB-23 is required for the formation of proper cell contacts between contralateral pairs of ventral epidermal cells. Tissue-specific rescue experiments reveal a function of VAB-23 in ventral neuroblasts that control the enclosure of the embryo by the overlaying epidermal cells. Finally, we provide evidence suggesting a role of VAB-23 in post-transcriptional gene regulation. We thus propose that VAB-23 regulates the expression of multiple secreted guidance cues in ventral neuroblasts that direct the migration of the overlaying epidermal cells. Members of the VAB-23 family may perform similar functions during morphogenesis in other metazoans.

Introduction

Morphogenesis refers to a variety of processes that create the shape of an organ or an entire organism during development. The execution of a particular cell fate involves cell–cell recognition, cell migration, attachment or fusion in order to generate proper tissue architecture and eventually a functional organ. In most cases, these developmental processes proceed normally. However, factors such as age, environmental influences and heritable genetic anomalies can lead to aberrant cell fates, morphogenesis and subsequent onset of disease.

The *Caenorhabditis elegans* (*C. elegans*) embryo is a widely used model to understand the intricacies of tissue morphogenesis and organ formation (Chisholm and Hardin, 2005; Marston and Goldstein, 2006). Morphogenesis of the embryo begins approximately 4–5 hours following the first cell division with the birth of the epidermal precursor cells on the dorsal surface of the embryo (Sulston et al., 1983). One of the first steps of morphogenesis is known as dorsal intercalation, in which two rows of ten epidermal cells wedge in between themselves, resulting in the formation of an epidermal sheet (Heid et al., 2001). Morphogenesis proceeds with ventral closure, where contralateral rows of epidermal cells migrate and extend along the sides of the embryo towards the ventral surface (Sawa et al., 2003). Upon converging on the ventral side, contralateral pairs of epidermal cells attach to each other and thereby seal the internal contents of the embryo. The process of ventral closure is dependent on the underlying neuroblasts, which act as a substrate to guide the epidermal cells towards the ventral side (Chin-Sang et al., 1999; George et al., 1998). The Ephrin tyrosine kinase receptor VAB-1 and its ligand VAB-2 are required in the neuroblasts for the sealing of the gastrulation cleft, which is necessary for the correct positioning of the ventral neuroblasts and the subsequent migration of the overlaying epidermal cells towards the ventral midline (Chin-Sang et al., 1999; George et al., 1998). In addition, MAB-20, the semaphorin-2A ortholog, prevents ectopic epidermal cell contacts during embryonic and post-embryonic development (Roy et al., 2000). Lastly, the embryo undergoes elongation via actin-mediated contractile forces shortly after the attachment of the epidermal cells on the ventral side has been completed (Diogon et al., 2007). During elongation, the embryo takes on the tube-like shape of the worm.

Several structural components required for morphogenesis of the embryo have been described. For instance, the attachments between epidermal cells require the HMR-1/HMP-1/-2 cadherin–catenin complex that forms adherens junctions (Costa et al., 1998) and the APC homolog APR-1 (Hoier et al., 2000). Other proteins such as AJM-1, DLG-1 and LET-413 form a distinct, apical cell junction complex that acts redundantly with the cadherin–catenin complex (Bossinger et al., 2001; Firestein and Rongo, 2001; Koppen et al., 2001; Legouis et al.,

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2000; McMahon et al., 2001). The subsequent elongation phase is dependent on the opposing actions of the myosin phosphatase MEL-11 and Rho kinase LET-502 (Diogon et al., 2007; Piekny et al., 2000).

In contrast to the relatively good knowledge of the structural determinants of embryonic morphogenesis, less is known about the transcriptional and post-transcriptional machinery involved. The C2H2 zinc finger transcription factor DIE-1 and the redundant T-box transcription factors TBX-8 and TBX-9 are necessary for dorsal intercalation and elongation (Heid et al., 2001; Pocock et al., 2004), while the homeodomain-containing proteins PAL-1 and UNC-62 are essential for proper morphogenesis of the posterior region (Edgar et al., 2001; Van Auken et al., 2002).

Here, we describe the functional characterization of the conserved nuclear Zinc finger protein VAB-23. VAB-23 prevents the formation of ectopic cell contacts between adjacent, ipsilateral ventral epidermal cells and mediates contact formation between contralateral pairs of cells across the ventral midline. Tissue-specific rescue experiments indicate that VAB-23 acts in the underlying ventral neuroblasts that guide the epidermal cells to the midline. Lastly, we present evidence for a possible function of VAB-23 in post-transcriptional gene regulation through the C-terminal Zinc finger domain.

Results

The vab-23 gene is essential for embryogenesis

The *vab-23* gene (annotated by the *C. elegans* Genome Sequencing Consortium as ZK930.3) was selected during an RNAi screen for regulators of vulval morphogenesis (M.W.P. et al., unpublished results). We chose the gene name *vab* because of the *v*ariable *ab*normal phenotype observed in *vab-23* mutants as shown below. Since we observed a strong protruding vulva (Pvl) phenotype in *vab-23* RNAi-treated animals (Suppl. Fig. S1), we wished to further analyze the developmental function of *vab-23*. For this purpose, we used the *vab-23 (tm1945)* deletion strain (kindly provided from the Japan Knockout Consortium), which eliminates 606 bp of the genomic open reading frame (Fig. 1A). The *tm1945* deletion produces a frameshift mutation and results in an early stop codon at the beginning of the coiled-coil region of VAB-23, indicating that this deletion likely represents a strong reduction-of-function or null allele (Fig. 1A). Using RT–PCR, we were able to amplify a cDNA corresponding to a truncated *vab-23(tm1945)* transcript (data not shown). The *vab-23(tm1945)* deletion resulted in a 100% penetrant zygotic lethal phenotype that could be rescued by germ line transformation of the entire *vab-23* genomic locus (Figs. 1A and 8A) (e.g., among the progeny of *vab-23(tm1945)*/+ heterozygous mothers, no *vab-23(tm1945)* homozygous larvae that had developed past the early L1 stage were found, $n = 1005$). In the following, we concentrate our analysis on the functions of *vab-23* during embryogenesis.

The gene prediction program Genefinder indicated the existence of two *vab-23* transcripts: A long 762 bp *vab-23a* transcript, which includes five exons, and a shorter 600 bp *vab-23b* transcript produced from an alternative promoter within the first intron (Fig. 1A) (www.wormbase.org; Stein et al., 2001). We used 5′ and 3′ Rapid Amplification of cDNA Ends (RACE) to confirm the predicted splicing pattern and isoform-specific primers to detect the two predicted *vab-23* transcripts. Reverse transcription (RT)–PCR experiments confirmed the existence of the major *vab-23a* transcript and of a less abundant *vab-23b* transcript starting at an alternative promoter in intron 1 and covering exons 2 to 5 (Suppl. Fig. S2). However, in transgenic animals carrying a rescuing translational GFP reporter construct containing both promoters, only one isoform corresponding to the predicted size of the VAB-23A::GFP fusion protein was detected by immunoblotting (Figs. 1A and B). Moreover, a translational *vab-23B::gfp* transgene lacking promoter sequences of *vab-23a* (Fig. 1C) was unable to rescue the *vab-23*

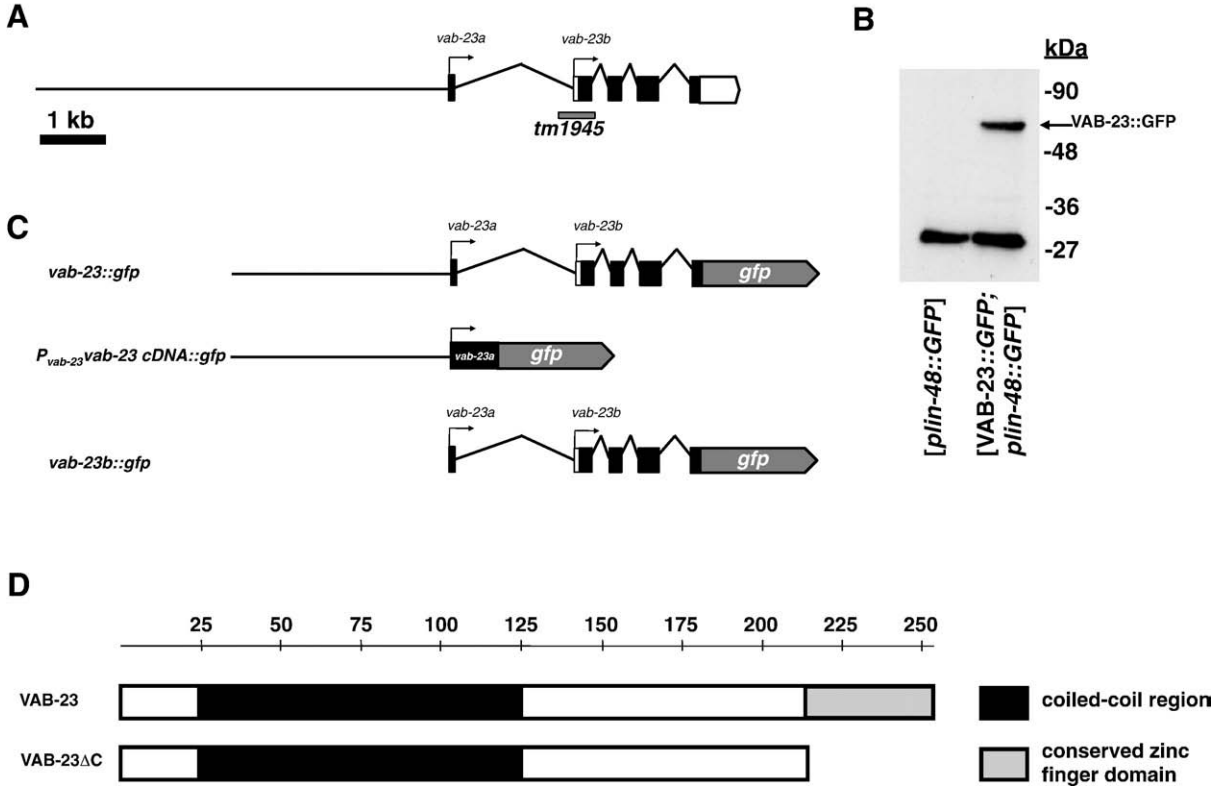

Fig. 1. *vab-23* encodes a coiled-coil protein. (A) Structure of the *vab-23* locus and the *vab-23::gfp* translational reporter used. The gray bar indicates the location of the *vab-23 (tm1945)* deletion. (B) Anti-GFP Western blot of total lysates from transgenic animals carrying either the *plin-48::gfp* transformation marker alone (left lane) or together with the *vab-23::gfp* reporter (right lane). The bands at approximately 30 kDa and 60 kDa correspond to GFP and the VAB-23::GFP fusion protein, respectively. (C) Structures of the *vab-23* rescue and reporter constructs used in this study. (D) Domain structure of the wild-type VAB-23 and the truncated VAB-23ΔC proteins.

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mutant phenotypes described below (data not shown). We thus conclude that the VAB-23A protein produced by the long *vab-23a* transcript is the major isoform, whereas the protein encoded by the shorter *vab-23b* transcript was below our detection limit. Expression of a *vab-23a* minigene was sufficient to rescue the *vab-23(0)* mutant phenotypes (Figs. 1C and 8A). Thus, the long *vab-23a* isoform (referred to as *vab-23* hereafter) is both necessary and sufficient to carry out the essential functions of *vab-23* during embryogenesis.

VAB-23 belongs to a novel family of coiled-coil proteins conserved in metazoans

The VAB-23 protein consists of 253 amino acids with a predicted molecular weight of 29 kDa (Fig. 1D). Protein sequence analysis of VAB-23 predicts two putative coiled-coil domains from amino acids 24 to 104 and 106 to 126 and a putative C4H2 zinc finger domain at the C-terminus (Fig. 2A). A potential nuclear localization signal (NLS)

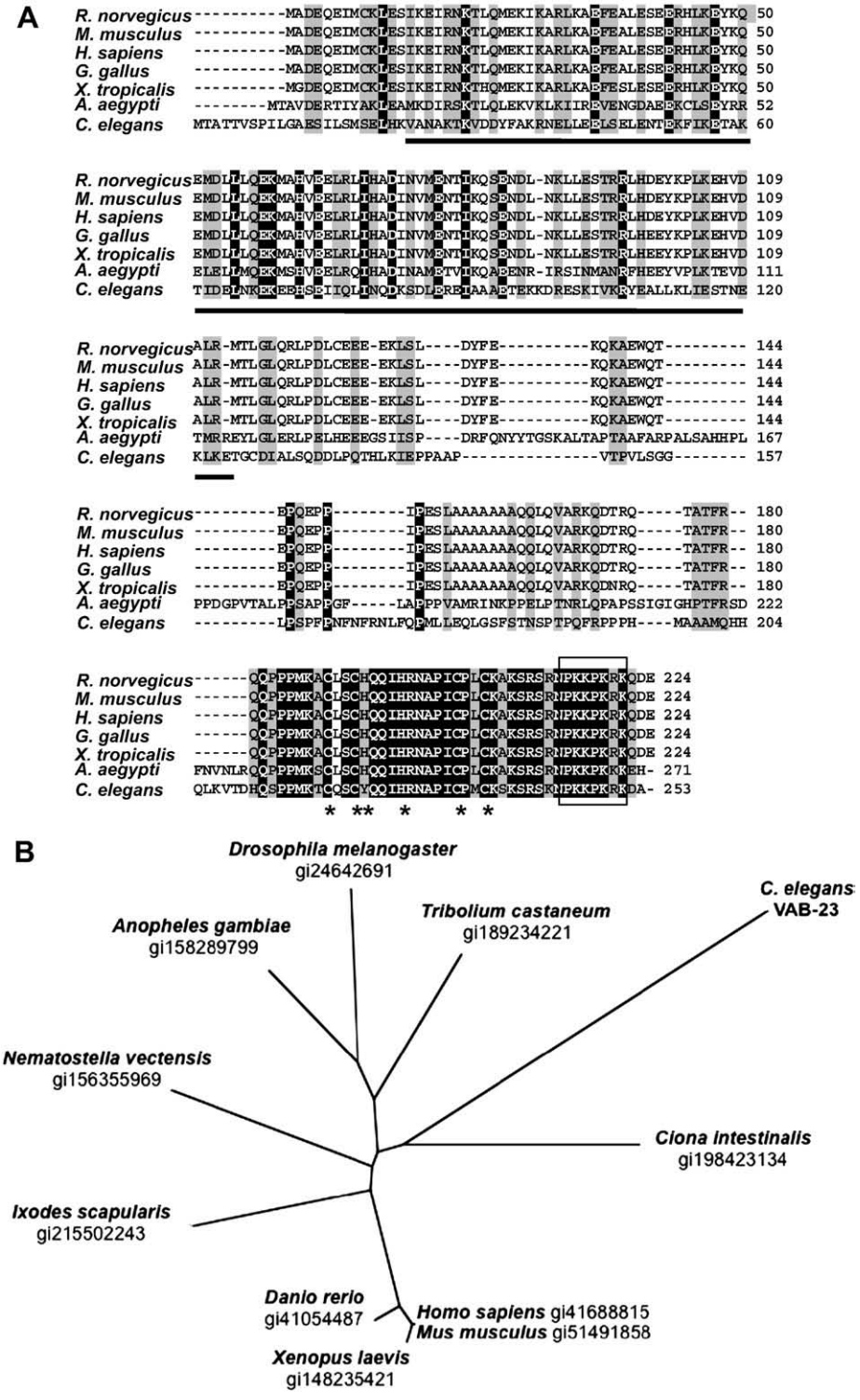

Fig. 2. VAB-23 is conserved in metazoans. (A) Alignment of *C. elegans* VAB-23 with its orthologs. The coiled-coil region of *C. elegans* VAB-23 is underlined. The predicted C4H2 zinc finger domain is marked with asterisks. The predicted NLS sequence is boxed. Similar and identical amino acids are shaded grey and black, respectively. (B) Unrooted tree diagram indicating the similarity between the different metazoan VAB-23 orthologs.

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is located between amino acids 245 and 251. The gene ZK930.2, which is located immediately adjacent to *vab-23*, exhibits close similarity to VAB-23 at the protein level and is likely the result of a recent gene duplication event (data not shown). While similar at the protein level, the gene products of *vab-23* and ZK930.2 are unlikely to be functionally redundant since loss of VAB-23 alone already leads to a completely penetrant lethal phenotype (see below).

C. elegans VAB-23 shows strong homology to a human hepatocellular carcinoma antigen (HCA127) of unknown function (Fig. 2A) (Wang et al., 2002). Except for *C. elegans*, all metazoan genomes analyzed encode a single VAB-23 ortholog (Fig. 2B). The coiled-coil domains of *C. elegans* and human VAB-23 possess approximately 25% sequence identity and 35% similarity, while amino acids 212 to 253 at the carboxy-terminus display the highest degree of conservation (80% identity). This most conserved C-terminal domain includes the putative C4H2 zinc finger domain that is characteristic for all members of this protein family. Even though VAB-23 belongs to a conserved protein family existing probably in all metazoans, no functional analysis has been reported for any of the VAB-23 orthologs in other species.

VAB-23 is necessary for ventral closure and elongation during morphogenesis of the embryo

We next investigated the role of *vab-23* during embryogenesis. Homozygous *vab-23(tm1945)* mutants segregated by *vab-23 (tm1945)/+* heterozygous mothers arrested with variable morphology defects either before embryonic elongation or shortly after hatching (Figs. 3C, G, and K). Epidermal morphogenesis in the wild-type embryo consists of three major events (Chisholm and Hardin, 2005; Marston and Goldstein, 2006). (1) During dorsal intercalation, the two contralateral rows of dorsal-most epidermal cells interdigitate and then fuse to form the dorsal epidermis. (2) During the subsequent ventral enclosure, the two contralateral rows of ventral epidermal cells migrate towards the ventral midline and form new cell junctions with their contralateral partner cells across the ventral midline. (3) Once the embryo is fully enclosed by the epidermal cells, actomyosin contractile forces in the middle row of epidermal cells (the seam cell precursors) drive the elongation of the oval embryo into a tube-like extended shape. Visualization of the epidermal cells in *vab-23(tm1945)* mutant embryos using the AJM-1::GFP apical junctional marker demonstrated various defects during epidermal morphogenesis (Mohler and White, 1998). In around 30% *vab-23(tm1945)* embryos (i.e., 5 out of 17 embryos, see below), ventral enclosure was defective, resulting in the extrusion of internal tissue (Figs. 3C and D). Embryos that were able to enclose arrested just prior to or during the elongation phase in around 50% of the cases (9 out of 17) or hatched with severe morphological deformities and arrested as L1 larvae in around 20% (3 out of 17) of the cases (Figs. 3G, H, K, and L). Interestingly, the morphological defects in *vab-23(tm1945)* mutants that arrested as L1 larvae were restricted to the posterior region (Fig. 3L). We observed no changes in the number of epidermal cells in *vab-23(tm1945)* embryos expressing AJM-1::GFP. In addition, although grossly malformed, *vab-23(tm1945)* embryos were able to twitch, indicating proper muscle differentiation. Therefore, the lethality of *vab-23(tm1945)* mutants likely results from defects in epidermal morphogenesis rather than cell fate changes.

VAB-23 is required for the formation of contacts between contralateral pairs of ventral epidermal cells

We next employed 4D time-lapse microscopy using AJM-1::GFP as an epidermal marker to examine whether the *vab-23(tm1945)* deletion affects cell adhesion *per se* or whether a defect in the ventral

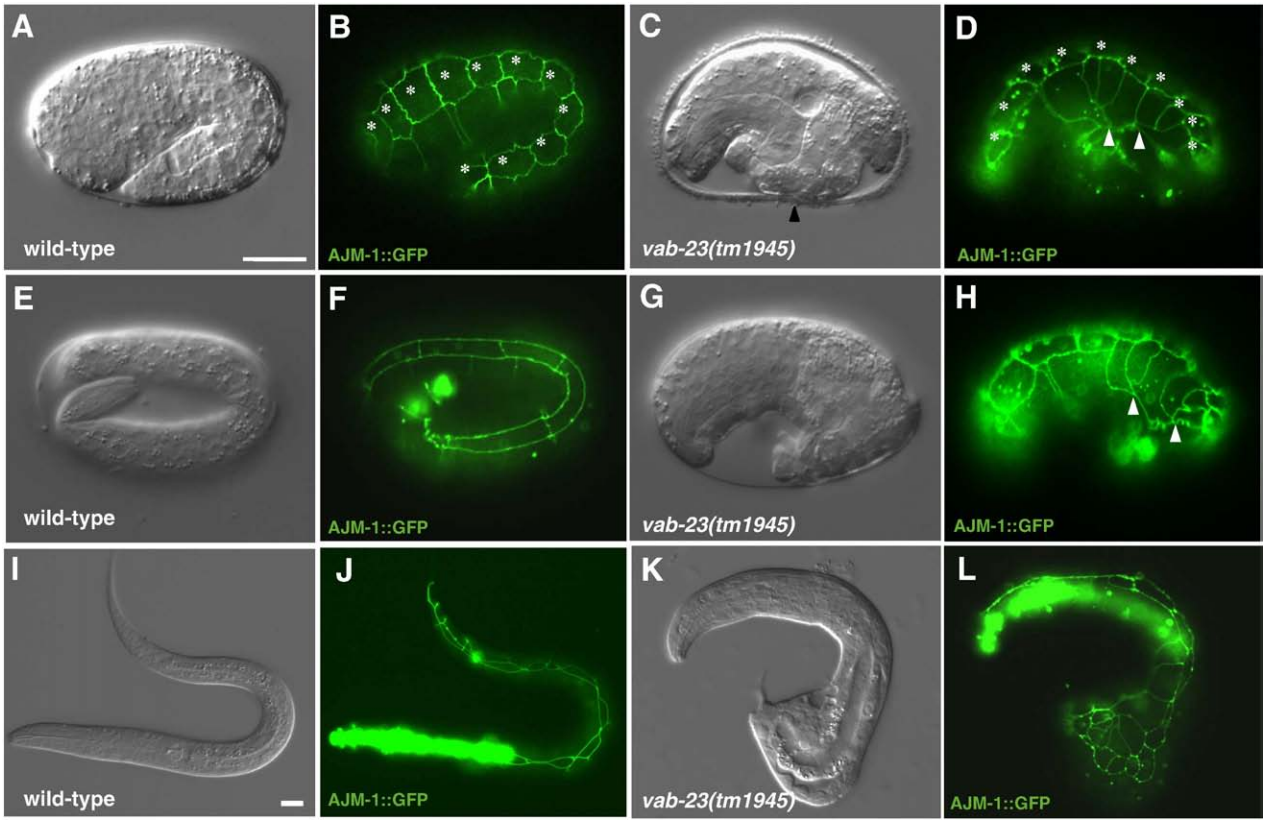

Fig. 3. VAB-23 regulates epidermal morphogenesis during embryogenesis. (A, B) Nomarski and corresponding AJM-1::GFP images of wild-type embryos during ventral enclosure, (E, F) elongation and (I, J) post-hatching. (C, D), (G, H), (K, L) Nomarski and AJM-1::GFP pictures of *vab-23(tm1945)* embryos at similar stages as in (A) through (J). Asterisks in (B) and (D) denote the lateral seam cells, the black arrowhead in (C) denotes the extruded gut contents due to failed ventral enclosure, and the white arrowheads in (D) and (H) point at two ventral epidermal cells making ectopic cell contacts on the same side of the midline. Anterior is to the left and dorsal up for all embryos shown. The scale bar in (A) and (I) is 10 μm.

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migration of the epidermal cells was the primary cause of the lethality. Dorsal intercalations appeared normal in all 17 *vab-23 (tm1945)* embryos observed (data not shown). Furthermore, no defects in the migration and attachment of the anterior leading cells were observed, and the initial migration of the posterior ventral epidermal cells (referred to as the ventral pocket cells) appeared superficially normal in the majority of embryos examined (Figs. 4A and B). However, ventral epidermal cells failed to form proper junctions with their contralateral partner cells across the midline in 5 out of 17 *vab-23(tm1945)* embryos resulting in an incomplete closure of the ventral cleft (Figs. 4B and C; Suppl. Movies 1 and 2). We consistently observed ventral epidermal cells making ectopic contacts with adjacent cells on the same side of the midline rather than making contact with their contralateral partner cells across the midline. Interestingly, the cell extensions made by the ventral epidermal cells that sought to make new contacts appeared misguided, and they often failed to cross the ventral midline (arrowheads in Fig. 4C and arrows in Suppl. Movie 2). In 9 out of 17 *vab-23(tm1945)* embryos, ventral closure appeared normal with only a few ectopic cell contacts made by the ventral pocket cells (Suppl. Movie 3). However, in these embryos, the contralateral pairs of epidermal cells in the mid-body region had not met at the midline by the time elongation started and the ventral cleft remained open, resulting in a developmental arrest later during elongation. Epidermal morphogenesis in the remaining three *vab-23(tm1945)* embryos observed by 4D microscopy appeared superficially normal, and they were able to elongate but arrested soon after hatching with posterior deformations (Fig. 3L).

We conclude that VAB-23 is required during ventral closure for the formation of proper cell contacts between contralateral pairs of ventral epidermal cells across the ventral midline.

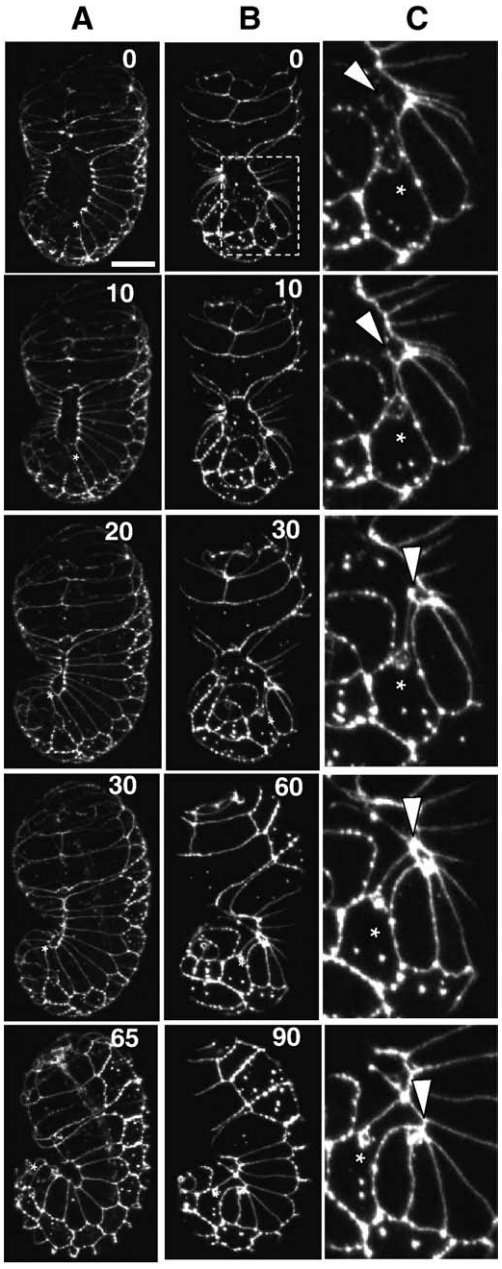

Fig. 4. Ectopic cell contacts are formed between ventral epidermal cells in *vab-23 (tm1945)* embryos. (A) Time-lapse recordings of wild-type and (B) *vab-23(tm1945)* embryos carrying the AJM-1::GFP marker starting at the time when the anterior leading cells have paired. The numbers in each panel of (A) and (B) indicate the elapsed time in minutes. Note that the *vab-23(tm1945)* embryo in (B) elongated more slowly. (C) Higher magnification of the region indicated by a dashed box in (B). The asterisks in (A) through (C) label the posterior-most ventral epidermal cells on one side that made ectopic contacts in the *vab-23(tm1945)* embryo (B) and (C) with more anterior epidermal cells. The arrowheads in (C) point at the cell extensions that resulted in the formation of inappropriate contacts between multiple cells on the same side of the midline resulting in a bright spot of the AJM-1::GFP signal. The scale bar in (A) is 10 μm.

VAB-23 function is required in neuroblasts to regulate epidermal morphogenesis

In order to examine the expression pattern of VAB-23 during embryogenesis, we used the *vab-23::gfp* translational reporter (Fig. 1A). This transgene rescued the morphogenesis defects observed in *vab-23 (tm1945)* embryos, demonstrating proper expression and functionality of the VAB-23::GFP fusion protein (Fig. 8A). VAB-23::GFP expression was observed at the onset of epidermal morphogenesis with predominant expression on the ventral posterior surface of the embryo (Figs. 5A–C). Expression continued throughout morphogenesis (Figs. 5D–F). Based on their position near the midline and the very similar expression pattern of another ventral neuroblast markers (see Fig. 6 and below), the majority of VAB-23::GFP expressing cells at the time of ventral closure are most probably ventral neuroblasts. In addition, at least two posterior ventral epidermal cells also expressed VAB-23::GFP (Figs. 5G–I, indicated with arrowheads).

Some of the previously characterized regulators of epidermal morphogenesis such as *vab-1* and *vab-2* have been shown to be required in the underlying neuroblasts that form the substrate on which the epidermal cells migrate (Chin-Sang et al., 1999; George et al., 1998; Ghenea et al., 2005; Harrington et al., 2002). Since VAB-23 was predominantly expressed in the ventral neuroblasts during ventral closure, we determined the tissue(s) in which *vab-23* acts during epidermal morphogenesis. Various promoters were used to drive the expression of the *vab-23* cDNA in the epidermis (P_{ajm-1}*::vab-23::gfp*, P_{dpy-7}*::vab-23::gfp*), in neuroblasts ($P_{unc-119}$*::vab-23::gfp*, P_{unc-33}*::vab-23::gfp*, P_{rgef-1}*::vab-23::gfp*) or in muscle cells (P_{myo-3}*::vab-23::gfp*). These transgenes were assayed for their ability to rescue the lethality of *vab-23(tm1945)* embryos (Table 1). Expression of *vab-23* cDNA under the control of the *unc-119* promoter that drives expression predominantly in neuroblasts and also some epidermal cells in the embryo (Maduro and Pilgrim, 1995; Hardin et al., 2008) or using the neuron-specific *unc-33* promoter (Altun-Gultekin et al., 2001) rescued the *vab-23(tm1945)* lethality to produce viable and fertile adults. On the other hand, expression of VAB-23::GFP in the epidermis or in muscle cells failed to rescue (Table 1). Interestingly, expression of *vab-23* under the *rgef-1* promoter also failed to rescue the *vab-23 (tm1945)* lethality. Although *rgef-1* expression is pan-neuronal, its expression does not precede embryonic epidermal morphogenesis but rather begins at the late comma stage of embryogenesis (Altun-Gultekin et al., 2001). Thus, VAB-23 acts in the ventral neuroblasts to guide the migration of the overlaying epidermal cells prior to or at the time of epidermal ventral closure.

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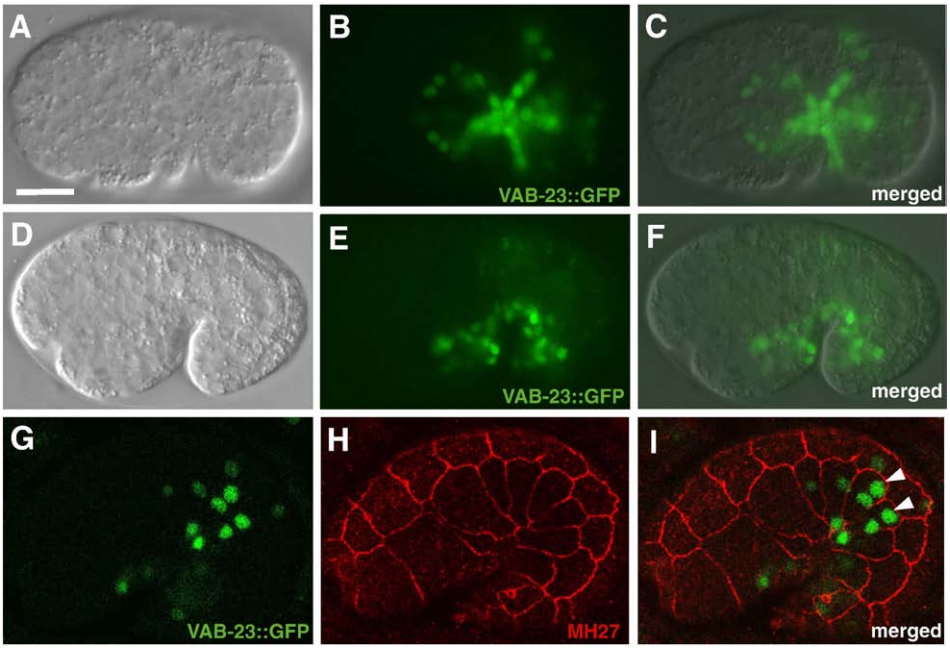

Fig. 5. VAB-23::GFP is expressed in both epidermal and neuronal precursor cells of the embryo. (A–C) Nomarski, VAB-23::GFP and merged images, respectively, of a bean stage embryo (ventral view) before and (D–F) a comma stage embryo shortly after ventral closure. (G) Single confocal section of a VAB-23::GFP embryo stained with anti-GFP (green) and (H) MH27 adherens junction (red) antibodies. (I) merged images of (G) and (H). Arrowheads in (I) point at 2 posterior ventral epidermal cells expressing VAB-23::GFP. Anterior is to left for all embryos shown and dorsal is up in (D) through (I). The scale bar in (A) is 10 μm.

The ventral neuroblasts move after gastrulation towards the ventral midline to seal the gastrulation cleft and form a substrate for the migrating epidermal cells during the subsequent ventral epidermal closure (Chisholm and Hardin, 2005). Mispositioning of the ventral neuroblasts as observed, for example, in *kal-1* mutants prevents proper contact formation between the contralateral epidermal cells during the subsequent epidermal morphogenesis and results in a phenotype similar to the one observed in *vab-23(tm1945)* mutants (Hudson et al., 2006). We therefore examined the positioning of the ventral neuroblasts using the pan-neuronal *unc-119p::gfp* reporter (Maduro and Pilgrim, 1995) or the *kal-1::gfp* reporter that is expressed predominantly in ventral neuroblasts at the time of ventral closure (Rugarli et al. 2002). We observed no obvious differences in the number or positioning of the *unc-119p::gfp* or *kal-1::gfp* positive ventral neuroblasts at the onset of epidermal enclosure in *vab-23 (tm1945)* embryos, although we cannot exclude the possibility of a slightly delayed enclosure of the gastrulation cleft in *vab-23(tm1945)* mutants (Fig. 6 and data not shown).

VAB-23 localizes to nuclear speckles and requires the zinc finger domain for its function

The VAB-23::GFP fusion protein was localized almost exclusively to the cell nuclei. Interestingly, the subnuclear localization of VAB-23::GFP was not uniform but was rather enriched in discrete, rapidly moving punctate (Fig. 7A), which is characteristic of proteins involved in pre-mRNA processing or mRNA regulation (Lamond and Spector, 2003). One hallmark of proteins involved in mRNA biosynthesis is their localization to so-called nuclear speckles that are found in interchromatin regions. To further examine the subnuclear localization of VAB-23::GFP, embryos were co-stained with the DNA-binding dye DAPI. This experiment revealed that VAB-23::GFP-containing

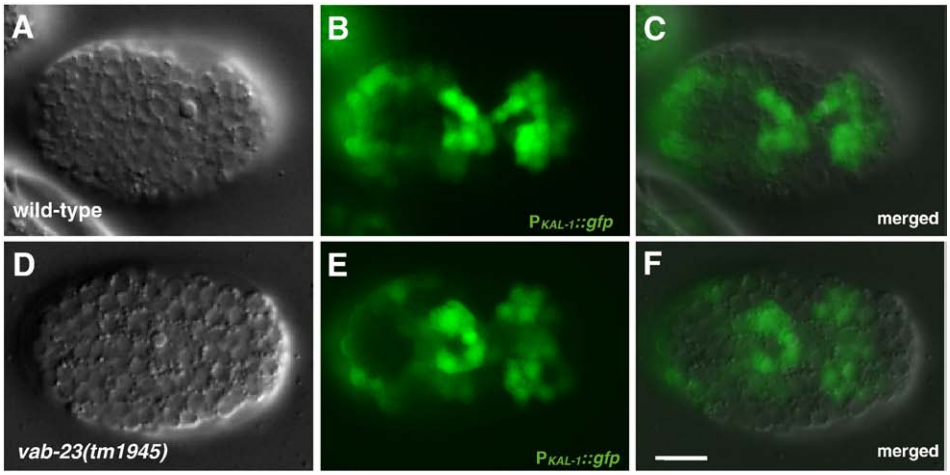

Fig. 6. Normal positioning of the ventral neuroblast positioning in *vab-23* mutants. Nomarski, GFP and merged images of wild-type and *vab-23(tm1945)* embryos carrying a *kal-1::gfp* transcriptional reporter (Rugarli et al., 2002). Ventral views of embryos at the onset of ventral closure (around 300–350 minutes after the first cleavage at 20 °C) are shown. Anterior is to the left for all embryos. Scale bar in (F) is 10 μm.

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Table 1
Tissue-specific rescue of *vab-23* embryonic lethality.

Promoter used for *vab-23* cDNA expression	Embryonic expression pattern	No. rescued lines/total lines analyzed
vab-23	Ventral neuroblasts, epidermis	3/3
ajm-1	Epidermis	0/5
dpy-7	Epidermis	0/12
unc-119	Early–late neuroblasts, epidermis[a]	6/6
unc-33	Early–late neuroblasts	8/9
rgef-1	Late neuroblasts	0/4
myo-3	Muscle	0/4

The indicated promoters were used to express *vab-23* cDNA with a C-terminal *gfp* tag and assayed for their ability to rescue the *vab-23(tm1945)* lethality (see Materials and Methods). The numbers of independent transgenic lines that showed rescue per total lines analyzed are indicated.
[a] See Hardin et al. (2008).

punctate do indeed represent regions of lower DNA content (Fig. 7B). We next tested the requirement of the conserved zinc finger domain at the C-terminus of VAB-23. This domain is highly conserved among most, if not all, metazoans. Interestingly, a H223Y substitution within the predicted C4H2 zinc finger domain is present only in *C. elegans* VAB-23. We therefore assayed the ability of a truncated version of VAB-23::GFP fusion protein that lacks 43 amino acids of the conserved C-terminus (referred to as VAB-23ΔC; Fig. 1D) to rescue the *vab-23(tm1945)* lethality. While the full-length VAB-23::GFP protein efficiently rescued the lethality of *vab-23(tm1945)* mutants, VAB-23ΔC::GFP was unable to restore VAB-23 activity (Fig. 8A). The inability of VAB-23ΔC::GFP to rescue could be due to a loss of nuclear localization, since a predicted NLS sequence resides in the conserved C-terminal region that was deleted. However, VAB-23ΔC::GFP was still localized to the nucleus similar to full-length VAB-23::GFP (Fig. 8B). Therefore, the highly conserved C-terminal zinc finger domain of VAB-23 is necessary for VAB-23 function, irrespective of its nuclear localization.

Discussion

We have identified the conserved VAB-23 protein as an essential regulator of epidermal morphogenesis in the *C. elegans* embryo. VAB-23 is necessary for the formation of proper cell contacts between contralateral pairs of epidermal cells across the ventral midline during ventral enclosure of the embryo. The observation of multiple ectopic cell contacts between ventral epidermal cells in *vab-23* mutants suggests that VAB-23 functions mainly by preventing contact formation between adjacent, ipsilateral epidermal cells. Since *vab-23* acts in the underlying neuroblasts and it encodes a nuclear protein, VAB-23 likely regulates the expression of specific target genes that act as secreted cues regulating different steps of morphogenesis. The localization to nuclear speckles and essential role of the zinc finger domain suggest that VAB-23 may function in the post-transcriptional regulation of target genes controlling epidermal morphogenesis, though we cannot exclude a role in transcriptional regulation. The direct downstream targets of VAB-23 are currently unknown, even though several secreted effectors of epidermal morphogenesis have been identified in *C. elegans*. For example, mutations in *mab-20*, which encodes a semaphorin-2A ortholog, cause similar ectopic cell contacts between epidermal cells, resulting in embryos with defective ventral enclosure and hatched larvae with body deformations (Roy et al., 2000). The two other semaphorin genes, *smp-1* and *smp-2*, are necessary for proper epidermal cell positioning and adhesion during larval development, but they are not expressed in ventral neuroblasts during morphogenesis of the embryo (Ginzburg et al., 2002). Furthermore, the *C. elegans* Kallman syndrome homolog *kal-1* acts in the underlying neuroblasts to regulate the formation of epidermal cell contacts during embryogenesis (Hudson et al., 2006). It is therefore tempting to speculate that VAB-23 might regulate *mab-20* or *kal-1* expression in ventral neuroblasts to prevent adhesion between adjacent ispilateral cells. Even though we did not observe a regulation of a transcriptional *kal-1* reporter by VAB-23, we cannot exclude the possibility that VAB-23 might regulate *kal-1* at a post-transcriptional level. Nonetheless, since null mutations in *mab-20* and *kal-1* only result in partial embryonic lethality and cause milder phenotypes than *vab-23* mutations, it is unlikely that misregulation of a single VAB-23 target is responsible for the completely penetrant lethality and strong morphogenesis defects seen in *vab-23* mutants. Our results rather indicate that *vab-23* controls the expression of multiple effector genes required for proper morphogenesis of the embryo.

The *vab-1* and *vab-2* genes, which encode an ephrin receptor tyrosine kinase and an ephrin ligand, respectively, also function in the ventral neuroblasts to regulate ventral enclosure of the embryo (Chin-Sang et al., 1999; George et al., 1998). It is unlikely that VAB-23 acts in an ephrin signaling pathway for the following two reasons: First, mutations in *vab-1* or *vab-2* rarely cause the formation of ectopic epidermal cell contacts as described here for *vab-23* and reported

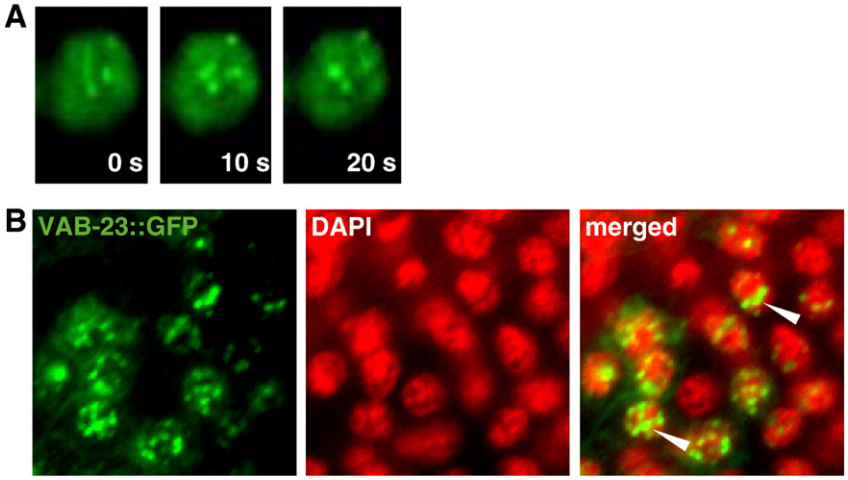

Fig. 7. VAB-23::GFP localizes to dynamic nuclear punctate. (A) Nuclear localization of VAB-23::GFP in epidermal cells of the larva showing dynamic nuclear punctate changing position over time. Images were acquired every 10 seconds. (B) Co-staining of VAB-23::GFP (green) with DAPI (red). Arrowheads indicate VAB-23::GFP punctate in regions of lower DNA content.

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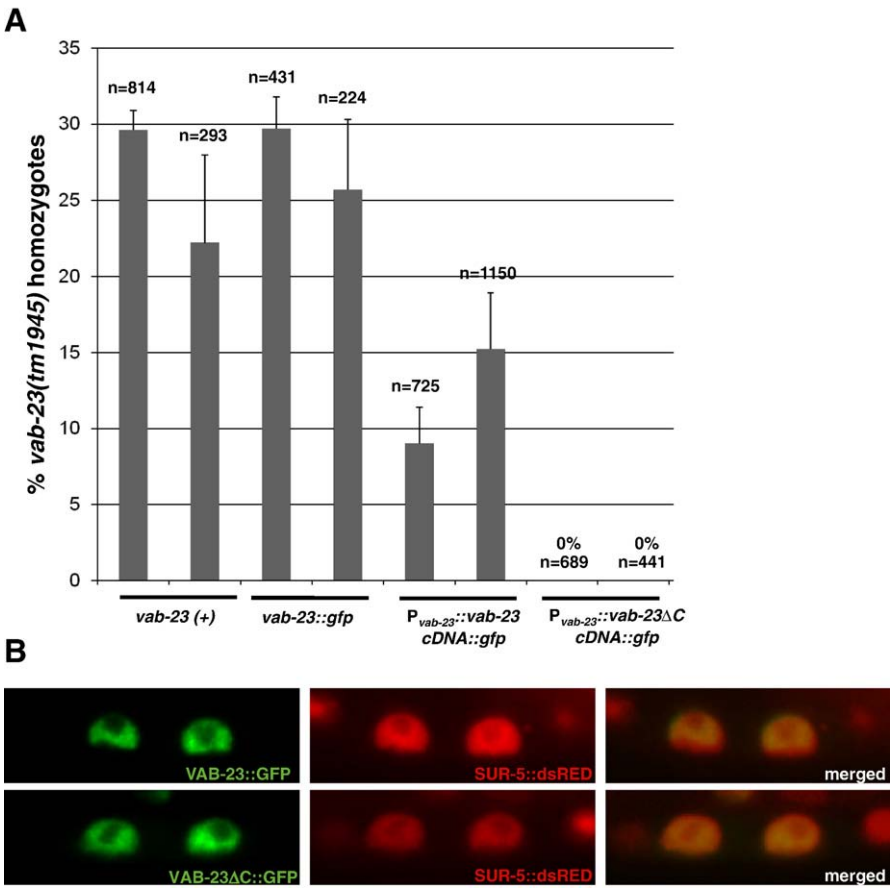

Fig. 8. The conserved C-terminus of VAB-23 is functionally necessary but not required for nuclear localization. (A) Rescue of the *vab-23(tm1945)* embryonic lethality. Two independent transgenic lines were assayed per construct as described in the Materials and Methods. *vab-23(+)* refers to a 14 kbp genomic fragment covering the entire *vab-23* genomic locus and *vab-23::gfp* to the translational reporter shown in Fig. 1A. $P_{vab\text{-}23}$*::vab-23 cDNA::gfp* refers to a *vab-23::gfp* translational reporter containing the *vab-23* cDNA fused at the C-terminus to *gfp*. Errors bars represent the standard error of the mean and were calculated from at least three broods. (B) *vab-23::gfp* and *vab-23ΔC::gfp* were co-transformed with a *sur-5::dsRed* translational reporter, which localizes to the nucleus. Both VAB-23::GFP and VAB-23ΔC::GFP proteins localized to the nucleus in speckles.

previously for *mab-20* mutants (Roy et al., 2000). Rather, VAB-1 and VAB-2 are required for the closure of the gastrulation cleft by the ventral neuroblasts. Second, *vab-1* and *vab-2* mutants that escape the embryonic lethality and arrest as L1 larvae display predominantly anterior morphology defects (i.e., a notched head phenotype) but do not exhibit strong posterior body defects as observed in arrested *vab-23* L1 larvae. It thus appears that the ephrin pathway and *vab-23* constitute two different systems regulating distinct aspects of epidermal morphogenesis in the anterior and posterior regions of the embryo, respectively.

The characteristic localization of VAB-23::GFP to dynamic nuclear speckles is reminiscent of two other *C. elegans* proteins that also localize to nuclear speckles and are important for neuronal development, the RRM-containing RNA binding protein UNC-75 and the C2H2 zinc finger protein SYD-9 (Loria et al., 2003; Wang et al., 2006). It is possible that VAB-23 functions similar to UNC-75 or SYD-9 as a post-transcriptional regulator that binds to specific mRNA targets with its conserved C-terminal zinc finger domain that is essential for VAB-23 function but not for nuclear localization. It will therefore be of interest to investigate whether VAB-23 does indeed act as a post-transcriptional regulator of morphogenesis and to identify mRNAs that might be bound by VAB-23.

Given the strong similarity between the VAB-23 orthologs in other species, it seems likely that this protein family plays an essential and conserved role during metazoan development. Consistent with this notion, a transposon insertion in the zebrafish *vab-23* ortholog has been identified in a genetic screen for genes essential for early development (Amsterdam et al., 2004). Our study provides the first insight into the function of this conserved and essential protein family and a framework for future investigation.

Materials and methods

General methods and strains

C. elegans strains were maintained at 20 °C on standard nematode growth media as described previously (Brenner, 1974). The wild-type strain of *C. elegans* used was Bristol N2. Other strains used are as follows: LGII: *vab-23(tm1945)/mIn1[mIs14 dpy-10(e128)]* (this study), Extrachromosomal arrays (this study) and integrated arrays: *jcIs1[ajm-1::gfp], otIs33[kal-1::gfp], zhEx229[pgisb-1::gfp, plin-48::gfp], zhEx271[punc-119::vab-23::gfp, plin-48::gfp], zhEx275[pgisb-1::gfp, pRF4(rol-6d)], zhEx280[pmyo-3::vab-23::gfp, plin-48::gfp], zhEx290 [pvab-23::vab-23::gfp, plin-48::gfp, zhEx295[14kbvab-23, pTGred(sur-5::DsRed)], zhEx297[pajm-1.vab-23::gfp, plin-48::gfp], zhEx298[prgef-1. vab-23::gfp, plin-48::gfp], zhEx313[pvab-23B.VAB-23B::gfp, plin-48:: gfp], zhEx321[punc-33.vab-23::gfp, plin-48::gfp], zhEx322[pdpy-7.vab-23::gfp, plin-48::gfp], zhEx329[pvab-23.vab-23ΔC::gfp, sur-5::dsRed].*

All constructs were microinjected into the gonad arms of adult worms at varying concentrations to generate stable transgenic lines (Mello et al., 1991). Injected solutions contained the desired plasmid to be tested along with a coinjection marker. Concentrations of each test construct was between 10 and 30 ng/μl along with the coinjection markers *plin-48::gfp* or *sur-5::dsred* at 50–75 ng/μl and pBluescript added to a final concentration of 150–200 ng/μl.

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Molecular analysis of the vab-23 locus

5′ and 3′ rapid amplification of cDNA ends (RACE) were performed using the BD SMART™ RACE cDNA Amplification Kit (BD Biosciences Clontech) according to the manufacturer's instructions. Primers isb-1.RACE.R2 and isb-1.RACE.F were used for 5′ and 3′ RACE, respectively (primer sequences available upon request). RNA isolation from mixed-stage wild-type worms was performed using standard methods, and cDNA was synthesized using the Superscript™ III kit according to the manufacturer's instructions (Invitrogen). Standard reverse transcription–polymerase chain reaction (RT–PCR) was also used to confirm the predicted splicing patterns of *vab-23*, using wild-type N2 cDNA as a template and gene-specific primers to detect the predicted transcripts of *vab-23*. Primers OMP25 and OMP26 were used to amplify the predicted *vab-23a* cDNA and bind to predicted exons 1 and 5, respectively. Primers OMP118 and OMP119 were used to amplify the predicted *vab-23b* cDNA and bind to predicted intron 1 (containing the start of the predicted coding sequence of *vab-23b*) and intron 4, respectively. RACE and RT–PCR products were sequenced to confirm their identity. Primers OMP93 and OMP94 were used to amplify the full *vab-23* locus. The fosmid WRM-06-38-H17 (Geneservice) was used as a PCR template. Worm lysates for Western Blot analysis were prepared by lysing 100 L4s in Laemmli buffer and boiling for 5 minutes. Immunoblot analysis was performed using 1:1000 affinity-purified anti-GFP antibody (Roche) and standard methods.

VAB-23 translational GFP reporter constructions

A 3.4 kb genomic fragment covering the region upstream of the *vab-23* ATG was amplified by PCR and cloned into the *Sal*I and *Bam*HI sites of pPD95.75 yielding pZK930.3::GFP (details available upon request). The complete genomic open reading frame (ORF) of *vab-23* was then amplified and cloned into the *Bam*HI site of pZK930.3::GFP to produce the VAB-23 translational GFP reporter plasmid VAB-23::GFP. To create the *vab-23* minigene GFP plasmid P_{vab-23}.*vab-23*::GFP, the full-length cDNA of *vab-23* was PCR amplified and cloned into the *Bam*HI and *Sma*I sites of pZK930.3::GFP (details available upon request). For P_{vab-23}.*vab-23*Δ*C*::GFP, a truncated *vab-23* cDNA (i.e., deletion of the C-terminal 43 aa) was cloned into the *Bam*HI and *Sma*I sites of pZK930.3::GFP. $P_{vab-23B}$.*VAB-23B*::GFP was obtained by amplifying the complete genomic ORF of *vab-23* fused to GFP and *unc-54* UTR from VAB-23::GFP.

Immunostaining and microscopy

Immunostaining of *C. elegans* embryos was performed as described previously (Miller and Shakes, 1995). Embryos laid from gravid hermaphrodite adults were mounted on poly-lysine-coated slides in ~6–10 μl of M9 buffer (22 mM KH_2PO_4, 22 mM Na_2HPO_4, 85 mM NaCl, 1 mM $MgSO_4$). After covering the dissected worms with a glass coverslip, slides were frozen on dry ice for 5–7 minutes. Eggs were permeabilized using the freeze-crack method (Miller and Shakes, 1995) and immediately fixed in absolute methanol at −20 °C. Air-dried slides were blocked using 3% bovine serum albumin (BSA) (in PBS; 0.2 M phosphate, 1.5 M NaCl, pH 7.4) for 30 minutes and specimens were then incubated with primary antibody (1:25 MH27) for 2 h at room temperature. Slides were washed five times with PBS-T (0.1% Tween 20 in 1× PBS) and incubated with secondary antibody (1:100 anti-mouse TRITC) for 1 h at room temperature. Specimens were then stained with 1:1000 4′,6-diamidino-2-phenylindole (DAPI) and washed five times with PBS-T before mounting with Movial solution.

Fluorescent images were obtained using a Leica DMRA wide-field microscope, equipped with a cooled CCD camera (Hamamatsu ORCA-ER). Images were analyzed using Openlab 3.0 software package (Improvision). Four-dimensional (4D) GFP microscopy of living embryos was analyzed using an Olympus FV1000 confocal microscope. Images were captured every 5 minutes using stacks of 1 μm. Images were processed using the Imaris program.

Tissue-specific rescue experiments

To express *vab-23* in the epidermis, the regulatory sequence from *ajm-1* (Koppen et al., 2001) was amplified and fused to a PCR fragment containing the *vab-23* cDNA, GFP and *unc-54* UTR from P_{vab-23}.::*vab-23*::GFP using the fusion PCR technique (Hobert, 2002). For P_{dpy-7}::*vab-23*::GFP, the *vab-23* cDNA was first amplified and cloned into the *Bam*HI and *Sma*I sites of pPD95.75 creating pPD95.75.ZK930.3.cDNA. 424 bp of *dpy-7* promoter (Gilleard et al., 1997) was then amplified and cloned into the *Sal*I and *Bam*HI sites of pPD95.75.ZK930.3.cDNA. To construct $P_{unc-119}$::*vab-23*::GFP, approximately 3 kb of *unc-119* promoter were amplified and cloned into the *Sal*I and *Bam*HI sites of pPD95.75.ZK930.3.cDNA yielding $P_{unc-119}$.*vab-23*::GFP. For P_{unc-33}::*vab-23*::GFP, 2.9 kb of promoter sequence corresponding to *unc-33* were amplified and cloned into the *Sal*I and *Bam*HI sites of pPD95.75.ZK930.3.cDNA. A fusion PCR approach was used to construct P_{rgef-1}::*vab-23*::GFP, using 2.1 kb of promoter sequence from *rgef-1*. To construct P_{myo-3}.*vab-23*::GFP the *vab-23* cDNA was amplified and cloned into the *Kpn*I and *Bam*HI sites of pPD136.64. All transgenic strains were examined for expression of GFP using standard epifluorescent microscopy to confirm the expression of the transgenes. To quantify the rescuing capability of the *vab-23* transgenes, *vab-23(tm1945)/mIn1* transgenic parents were allowed to lay eggs for a period of 4 days. The rescuing capability is expressed as a percent ratio of the number of transgenic *vab-23(tm1945)* homozygous animals to the total number of transgenic progeny. Note that full rescue corresponds to a 25% ratio of *vab-23(tm1945)* homozygotes. All rescued transgenic lines shown in Table 1 and Fig. 8A segregated between 8% and 20% *vab-23(tm1945)* homozygotes, while nonrescued lines never segregated any *vab-23(tm1945)* homozygotes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2009.09.036.

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